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Streamlining methodology for the multiresidue analysis of β-lactam antibiotics in bovine kidney using liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

A previously reported multiresidue method for the analysis of 11 important β-lactams (amoxicillin, ampicillin, cefazolin, cephalexin, cloxacillin, desfuroylceftiofur cysteine disulfide (DCCD), deacetylcephapirin, dicloxacillin, nafcillin, oxacillin, and penicillin G) in bovine kidney has been further streamlined. The method is based on a simple extraction using acetonitrile–water (4:1, v/v), followed by dispersive solid-phase extraction clean-up with C_{18} sorbent, concentration of an extract aliquot, and filtration of the final extracts using syringeless filter vials, which are used for the sample introduction in the liquid chromatographic–tandem mass spectrometric (LC–MS/MS) analysis. The recoveries have been improved by adding the internal standard [$^{13}C_6$]sulfamethazine to the homogenized sample before the extraction step, which enabled a proper control of the volume changes during the sample preparation. Average recoveries of fortified samples were 87–103% for all β-lactams, except for DCCD, which had an average recovery of 60%. Based on the results of the stability study and LC mobile phase tests, methanol has been eliminated from the entire method, including the LC–MS/MS analysis. The best overall LC–MS/MS (electrospray positive ionization) performance was achieved by using 0.1% formic acid as an additive in both parts of the mobile phase, in water and in acetonitrile. To prevent carry-over in the LC–MS/MS analysis, the LC method was divided into two parts: one serving as an analytical method for injection of the sample and elution of the analytes and the other one, starting at a highly organic mobile phase composition, being dedicated for injection of a solvent, washing of the system, and equilibration of the column to the initial conditions of the analytical method. In this way, a blank solvent is injected after each sample, but these in-between injections contribute minimally to the overall sample throughput.

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1. Introduction

β-Lactam antibiotics are one of the most widely applied antimicrobial drugs in current veterinary practice. The class of β-lactams includes penicillins and cephalosporins that both have a β-lactam ring in their structures, but this ring is fused to a five-membered thiazolidine or a six-membered dihydrothiazine ring, respectively.

In the USA, bovine kidney is the target tissue for monitoring of β-lactam (and other antibiotics) levels in cattle within enforcement and surveillance programs of the Food Safety and Inspection Service (FSIS) of the US Department of Agriculture. Currently, the FSIS uses a semi-quantitative microbial assay to determine violative levels of β-lactams (and some other antibiotics) and employs liquid chromatography–tandem mass spectrometry (LC–MS/MS) only for identification/confirmation purposes. Our laboratory efforts aim at development of quantitative multiresidue method(s) for veterinary drug residues based on LC–MS/MS analysis and transfer of these methods to the FSIS and other laboratories.

Several LC–MS-based methods for the analysis of one or more β-lactams in bovine kidney have been previously reported [1–7]. Two of the multiresidue methods were developed in our laboratory by Fagerquist and Lightfield at first for confirmatory analysis using an ion trap MS instrument [3], which was later replaced by a triple quadrupole MS instrument for a more quantitative analysis [4]. Furthermore, the latter method employed dispersive solid-phase extraction (SPE) clean-up instead of the previously used cartridge-based SPE, which simplified and speeded up the sample preparation.

As opposed to microbial assays, LC–MS-based methods usually employ organic solvents (mainly methanol or acetonitrile) for sample preparation and analyte elution, which can pose problems in terms of analyte stability during the analytical process and standard solution/extract storage. Tyczkowska et al. [8] previously reported relatively rapid degradation of cloxacillin in methanol (MeOH) and

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MeOH–water solutions, presumably due to the methyl ester formation [9]. MeOH–water (50:50, v/v) was used for the preparation of β-lactam stock solutions in the above-mentioned methods [3,4]. Thus, a question arose, which was related to the use of MeOH in the β-lactam method, including not only standard preparation but also its use as a part of the mobile phase in the LC–MS/MS analysis, since MeOH is a frequently employed mobile phase solvent in β-lactam LC–MS methods [4,5,7,10–14].

In this study, we evaluated stability of β-lactams at different conditions in solutions prepared in solvents typically used in LC–MS-based methods for analysis of antibiotics. We also investigated the impact of the LC mobile phase composition (% addition of formic acid, presence of MeOH, etc.) on LC–MS/MS analysis sensitivity, selectivity, ruggedness, and speed for 14 β-lactams. Finally, we further improved the previously reported multiresidue method for the analysis of β-lactams in bovine kidney [4] by streamlining the procedure and by introducing a proper control of the volume changes during the sample preparation.

2. Experimental

2.1. Chemicals and materials

Reference standards, all 95% or higher purity, were obtained from US Pharmacopeia (Rockville, MD, USA); except for cefadroxil and propranolol (used as an internal standard), which were obtained from Sigma (St. Louis, MO, USA). Deacetylcephapirin was provided by the Center of Veterinary Medicine of the US Food and Drug Administration (Laurel, MD, USA) and by the European Union Community Reference Laboratory for Antimicrobial Residues in Food (Fougères, France). A metabolite of ceftiofur, desfuroylceftiofur cysteine disulfide (DCCD), was provided by Pharmacia (Kalamazoo, MI, USA) and Pfizer (New York, NY, USA). The isotopically labeled standard of phenyl-[$^{13}C_6$]sulfamethazine (90%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

Individual stock solutions (at about 2000 μg/mL) were prepared in water, except for cefazolin, in which case MeCN–water (50:50, v/v) was used. For the stability study, a composite stock standard solution containing 10 β-lactams (amoxicillin, ampicillin, cefazolin, cephapirin, cloxacillin, dicloxacillin, nafcillin, oxacillin, penicillin G, and penicillin V) was prepared at 100 μg/mL in water. For the evaluation of the LC mobile phase composition, a composite stock standard solution containing 13 β-lactams (the above 10 analytes and 3 additional compounds: cefadroxil, cephalexin, and deacetylcephapirin) was prepared at 100 μg/mL in water. This solution was diluted 1000-fold in water (to 100 ng/mL) and DCCD was added at 1000 ng/mL to prepare a test solution of 14 β-lactams for the mobile phase evaluation study. For the recovery studies validating the method for multiresidue analysis of β-lactams in bovine kidney, one composite stock standard solution (at 50 μg/mL in water) contained 11 β-lactams (amoxicillin, ampicillin, cefazolin, cephalexin, cloxacillin, DCCD, deacetylcephapirin, dicloxacillin, nafcillin, oxacillin, and penicillin G). Another composite stock standard solution (also at 50 μg/mL in water) contained [$^{13}C_6$]sulfamethazine (an internal standard) and two β-lactams (cefadroxil and penicillin V) used for quality control purposes (cefadroxil and penicillin V are generally not used in veterinary medicine). All stock standard solutions were stored in 1 mL portions in polypropylene tubes at −20 °C.

MeOH, MeCN, and hexane were high-purity grade solvents for residue analysis from J.T. Baker (Phillipsburg, NJ, USA) and Burdick & Jackson (Muskegon, MI, USA), respectively. Formic acid (FA) was obtained as a 98% solution for MS from Fluka (Buchs, Switzerland). Ultrapure water was obtained from a Barnstead water purification system (Dubuque, IA, USA). Liquid-headspace supplied nitrogen serving as nebulizer, curtain, and collision gas in LC–MS/MS was obtained from Air Products (Allentown, PA, USA). Bovine kidneys (checked to be β-lactam free) were obtained from a local grocery store. Sorbents tested for dispersive SPE included C_{18} from J.T. Baker (Phillipsburg, NJ, USA), primary secondary amine (PSA) from Varian (Harbor City, CA, USA) and graphitized carbon black (GCB) from Supelco (Bellefonte, PA, USA).

2.2. Stability study experiments

For the stability study experiments, test solutions of the 10 β-lactams were prepared in replicates at 100 ng/mL in water, MeCN, MeCN–water (50:50, v/v), MeOH, or MeOH–water (50:50, v/v). The same set of solutions was also prepared with addition of 0.1% FA. Propranolol was added as an internal standard to all solutions at 200 ng/mL. The test solutions were analyzed by LC–MS/MS immediately after their preparation (t = 0 h) and stored in different conditions: in dark and clear vials at room temperature and light, in dark vials in the refrigerator (+4 °C) and in polypropylene tubes in the freezer (−20 °C). The solutions stored in dark vials at room temperature were analyzed in 6 h intervals for 5 days. All solutions were analyzed every week for 8 weeks. Responses of analytes were compared to freshly prepared solutions.

2.3. LC–MS/MS conditions

LC–MS/MS analysis was performed using an Agilent 1100 LC system with a binary pump, autosampler, column heater (kept at 30 °C), and degasser (Agilent Technologies, Palo Alto, CA, USA) interfaced to an API 3000 triple quadrupole mass spectrometer (Applied Biosystems; Toronto, ON, Canada). Injection volume was 5 μL in the stability study to enable direct injection of analyte solutions in all tested solvents without affecting peak focusing of the early eluting analytes. Injection volume of 10 μL was used in all other instances when analytes where introduced in aqueous solutions (in the mobile phase evaluation studies and the analysis of kidney extracts). A Phenomenex Prodigy ODS3 column (150 mm × 3 mm; 5 μm particle size, 100 Å pore size), coupled to a C_{18} 4 mm × 3 mm guard column (both from Phenomenex; Torrance, CA, USA), was employed for the LC separation. The flow rate of the mobile phase was 300 μL/min. A Valco (Houston, TX, USA) divert valve was placed between the column outlet and MS source to eliminate the introduction of co-extracted matrix components into the MS instrument prior and after elution of β-lactams.

In the stability study, the binary mobile phase was composed of A, 0.1% FA in water and B, 0.1% FA in MeCN. A linear gradient, starting from 2% B and going to 100% B within 15 min followed by a hold at 100% B till 19.5 min, was used in the analytical method. Afterwards, a wash method was performed for the injection of MeCN to prevent carry-over between samples. The wash method started at 100% B (held for 8 min), followed by a fast ramp (within 0.5 min) to 2% B, which was held until 15.5 min to equilibrate the column for the next run. Thus, the total cycle time was adjusted to take 36 min (including two injections taking 0.5 min each) to analyze the 10 different test solutions evaluated in the stability study in 6 h. For the analysis of kidney extracts, a faster gradient in the analytical method was used, going from 2% B to 100% B in 10 min and holding at 100% B until 14.5 min. The wash method was also shorter: a 5-min hold at 100% B, followed by a fast ramp to 2% B, which was held until 10.5 min, resulting in a cycle time of 26 min.

In the mobile phase evaluation study, the percentage of FA in both mobile phase components A and B was varied (x = 0.4, 0.2, 0.1, 0.05, 0.025, 0.01, 0.005, and 0%, v/v). The mobile phase A was x% FA in water. The mobile phase B was x% of FA in (1) MeOH; (2)

Table 1
Compound-specific LC–ESI(+)-MS/MS conditions for the tested β-lactams

Analyte	t_R (min)	DP (V)	FP (V)	Precursor ion	Product ion	CE (V)	CPX (V)
Deacetylcephapirin	7.8	47	143	382	152	37	12
					226	27	16
Amoxicillin	8.0	25	129	366	349	13	10
					208	19	14
Cefadroxil	8.1	24	110	364	114	29	10
					208	15	16
Cephapirin	8.4	42	182	424	292	21	8
					152	31	14
DCCD	8.4	66	240	549	183	43	18
					241	29	18
Ampicillin	8.5	36	142	350	106	29	8
					192	23	14
Cephalexin	8.6	27	117	348	158	13	12
					174	19	14
Cefazolin	10.3	26	140	455	323	17	10
					156	23	12
Penicillin G	11.9	36	180	335	160	17	14
					176	17	12
Penicillin V	12.3	21	110	351	160	17	14
					114	45	8
Oxacillin	12.5	31	160	402	160	19	14
					243	17	8
Cloxacillin	12.8	31	160	436	277	19	8
					160	19	12
Nafcillin	12.9	36	160	415	199	19	14
					171	49	16
Dicloxacillin	13.4	31	150	470	160	19	12
					311	19	10

t_R, retention time; DP, declustering potential; FP, focusing potential; CE, collision energy; CXP, collision cell exit potential.

MeOH–MeCN (50:50, v/v); (3) MeCN. A linear gradient, starting from 2% B and going to 100% B within 10 min followed by a hold at 100% B until the elution of the last analyte (dicloxacillin), was used for each A and B combination. Then, the column was well equilibrated to the same initial conditions (for repetitive analyses, $n = 4$) or to the initial conditions for the next tested mobile phase composition.

The MS determination was performed in electrospray (ESI) positive mode combined with monitoring of the most abundant MS/MS (precursor → product) ion transitions (dwell time of 75 ms for each transition). The following MS conditions were used: entrance potential of 10 V, ionspray voltage of 4500 V, and ion source temperature of 525 °C. The curtain gas regulator was set at 40 psi with optimum relative setting (in the Analyst software) of 11. The nebulizer and collision gas regulators were set at 90 psi with optimum relative settings of 14 and 12, respectively. Table 1 gives compound-specific MS/MS conditions and retention times obtained using the mobile phase composition and gradient employed in the analysis of kidney extracts. For the internal standard propranolol, MS/MS transitions m/z 260 → 116 and m/z 260 → 183 were monitored in the stability study. For the internal standard [$^{13}C_6$]sulfamethazine, MS/MS transitions m/z 285 → 186, 124, and 114 were used in the analysis of kidney extracts.

2.4. Sample preparation method for the analysis of β-lactams in bovine kidney

The optimized sample preparation procedure entailed the following steps: (1) weigh 1 g of thoroughly homogenized bovine kidney sample into a 50-mL disposable polypropylene centrifuge tube (Corning, Lowell, MA, USA); (2) add 100 μL of 1 μg/mL composite standard solution of [$^{13}C_6$]sulfamethazine (serving as an internal standard to compensate for volume changes), penicillin V and cefadroxil (a penicillin and a cephalosporin, respectively, serving as quality control standards indicating potential problems in real analysis of unknown samples) in water; (3) add 2 mL water and 8 mL acetonitrile; (4) vortex briefly, shake vigorously for 5 min; (5) centrifuge at 3450 rcf (5000 rpm using a RT6000B centrifuge from Sorvall, Newtown, CT, USA) for 5 min; (6) decant the supernatant into a disposable polypropylene 15 mL tube (Corning, Lowell, MA, USA) with 500 mg of C_{18} sorbent; (7) vortex briefly, shake for 30 s; (8) centrifuge at 3450 rcf for 1 min; (9) place 5 mL aliquot of the supernatant into a graduated tube; (10) evaporate extract to <1 mL; (11) make up the volume to 1 mL with water; and (12) transfer the extract into the chamber of a Mini-UniPrep Syringeless Filter vial (Whatman, Florham Park, NJ, USA) and compress the filter (poly(vinylidene difluoride), PVDF, 0.45 μm) plunger to filter the extracts, which are then ready for LC–MS/MS analysis that is performed on the same day as the sample extracts were prepared.

3. Results and discussion

3.1. Evaluation of the mobile phase composition for LC–MS analysis of β-lactams

As mentioned in Section 1, MeOH is a very popular mobile phase solvent in LC–MS analysis of β-lactams in spite the potential for analyte degradation. In our stability study experiments

performed at room temperature in dark vials, very rapid degradation of all tested β-lactams (somewhat slower for cephalosporins) was observed in MeOH and a slower degradation rate in the 50:50 MeOH–water mixture (still about 40–60% of the tested analytes left in the 50:50 MeOH–water solutions even after 5 days of storage). On the other hand, very good stability was observed in water, MeCN, and MeCN–water solutions, which were also found to be suitable solvents for long-term storage of standard solutions at the lower tested temperatures.

Similarly to MeOH, FA is a frequently employed mobile phase modifier in LC–MS analysis of antibiotics, including β-lactams [3–6,10–13,15–17]. Wiese and Martin [18] previously reported degradation of penicillin G at lower pH conditions. In our stability study, the 0.1% addition of FA to water and other aqueous solutions caused rapid degradation of monobasic penicillins, especially penicillin G and nafcillin. Degradation of other tested penicillins (amoxicillin and ampicillin) and cephalosporins was less pronounced (still about 40% of these analytes left in the solutions even after 5 days in water with 0.1% FA at room temperature).

To test the effect of MeOH and FA presence in the mobile phase on analyte responses and method ruggedness, we designed experiments involving the use of MeOH, MeCN, and MeOH–MeCN (50:50, v/v) as the organic part of the LC mobile phase (part B). At the same time, we varied the percentage of FA (0–0.4%, v/v) in both parts of the mobile phase (mobile phase A was water with *x*% of FA).

Independent of the amount of FA in the mobile phase, MeCN provided overall better sensitivity for the tested β-lactams than MeOH. The early eluting analytes (from deacetylcephapirin to cephalexin) were less affected by the organic mobile phase component, but 2.2–4.1-fold higher maximum responses were achieved with MeCN versus MeOH for the later eluting analytes (from cefazolin to dicloxacillin). In the case of MeCN, highest overall sensitivity was obtained with 0.1% FA (0.2% FA was the optimum for MeOH), which also corresponded with the maximum sensitivity of the late eluting analytes. Early eluters were less affected by the different amounts of FA in the mobile phase. They overall gave the best responses at 0.01% FA addition, but signals of the late eluters were very low at these conditions. Thus, we found 0.1% FA in MeCN to provide the best overall sensitivity for LC–ESI(+)-MS/MS multiresidue analysis of β-lactams as compared to other tested mobile phase compositions. Fig. 1 demonstrates the above discussed results, comparing responses (peak heights) of an early eluting analyte deacetylcephapirin and a late eluting analyte cloxacillin obtained with different % FA in the aqueous and organic (MeCN, MeCN–MeOH, or MeOH) mobile phase components.

In terms of the speed of the analysis, MeCN provided the overall fastest analyte elution as compared to MeOH and MeOH–MeCN. Fig. 2 shows retention times obtained for the last eluting analyte (dicloxacillin) using different mobile phase compositions. A similar trend can be observed for all late eluting analytes. For FA concentrations in MeCN $\leq$ 0.05%, the retention times shifted dramatically to higher values. Thus, 0.1% FA addition also ensured good robustness because only very slight changes in analyte retention times (and also responses) would occur with a less accurate addition of 0.1% FA. Furthermore, lower FA concentrations ($\leq$0.05%) also led to deterioration of peak shapes (significant peak broadening) for cefazolin and DCCD, which shifted to longer retention times more readily than the other β-lactams, thus resulting in different elution orders (e.g., DCCD eluting after ampicillin and cephalexin) as the % FA decreased in the mobile phase.

To prevent carry-over in LC–MS analysis, analysts often inject a blank solvent after each (or a high-level) sample using the same LC method to wash the system (mainly the needle, injection port, and valve). This procedure, however, significantly reduces sample throughput. We divided the LC method into two parts. The first part served as an analytical method for injection of the sample and elution of the analytes. The LC gradient of the analytical method ends at 100% B and holds there until the last analyte is recorded by the MS instrument. The second method was a wash method that started at 100% B and served for injection of a solvent (MeCN), washing of the system, and equilibration of the column to the initial conditions of the analytical method. In this way, the in-between samples injections of blanks contribute minimally to the overall sample throughput.

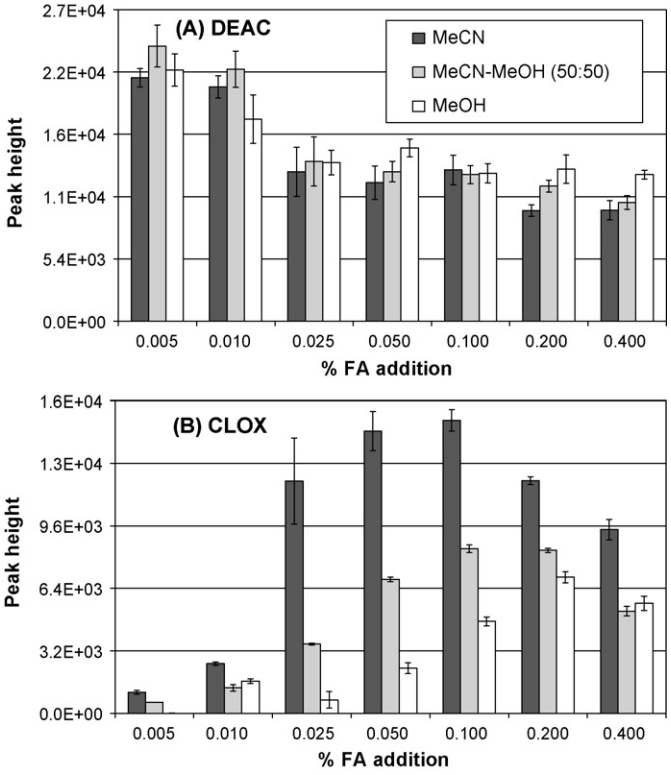

Fig. 1. Effect of the LC mobile phase composition on responses (peak heights) of (A) deacetylcephapirin and (B) cloxacillin using different amounts of FA (0.005–0.4%) in the mobile phase A (water) and B, which was MeOH or MeCN or MeCN–MeOH (50:50, v/v).

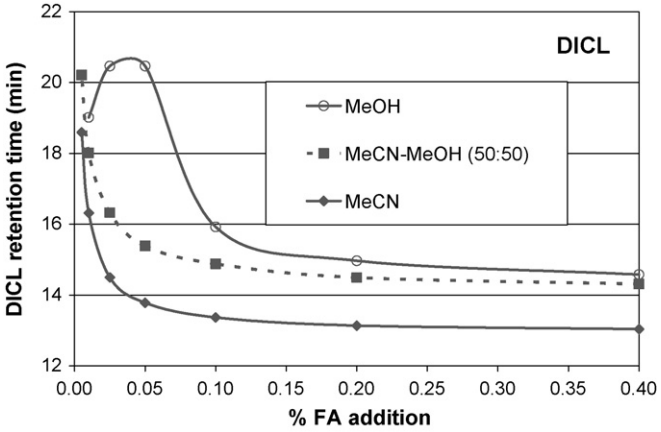

Fig. 2. Effect of the LC mobile phase composition on dicloxacillin retention time using different amounts of FA (0.005–0.4%) in the mobile phase A (water) and B, which was MeOH or MeCN or MeCN–MeOH (50:50, v/v).

3.2. Streamlined method for multiresidue analysis of β-lactams in bovine kidney

The previously reported sample preparation method [4] is based on the extraction of homogenized tissue with MeCN–water (4:1, v/v), followed by a dispersive SPE clean-up using octadecyl silica (C_{18}) as a sorbent. After the clean-up step, the extract is concentrated by evaporation, resulting in the final extract in water. The 4:1 MeCN–water mixture used as 10 mL per 1 g sample was found to be optimal for both β-lactam extraction and sample deprotenization [19].

Dispersive-SPE technique involves a simple mixing of the extract with a sorbent that removes matrix interferences but does not retain the analytes [20]. C_{18} sorbent removes highly lipophilic compounds [21] (we found 500 mg per 10 mL of the kidney extract to be an optimum in terms of clean-up and cost-effectiveness). Other effective sorbents applied in the dispersive format include primary secondary amine and graphitized carbon black that are employed mainly for removal of fatty acids and pigments (and some other matrix-coextractives), respectively. Unfortunately, PSA and GCB cannot be used in the analysis of β-lactams because they contain a carboxylic group that is being retained by PSA, resulting in lower analyte recoveries. GCB has a high affinity towards planar molecules, therefore also highly retains nafcillin and DCCD, which recoveries were only about 20–30% when 50 mg GCB was added to 1 mL of 100 ng/mL β-lactam solution in 4:1 MeCN–water.

In our effort to further streamline the method and make it more cost-effective, we also tested the use of hexane as an alternative to the dispersive C_{18} clean-up. Hexane is immiscible with MeCN and water, thus can be used for removal of lipophilic compounds from MeCN–water extracts [22]. In our evaluation, we added 5 mL of hexane to 10 mL of kidney extract, shaked well, and removed the upper hexane layer after a centrifugation step. Using the same extract, we also performed the dispersive-C_{18} clean-up and the hexane clean-up followed by the dispersive-C_{18} procedure. In each case, the amount of matrix co-extractives left as a residue after extract evaporation was determined gravimetrically and compared versus the situation without any clean-up. The dispersive-C_{18} clean-up removed about threefold more matrix co-extractives than hexane. Also, no additional clean-up was achieved using the combination of hexane and C_{18} as compared to C_{18} alone.

A quantitative limitation of the previously introduced method [4] was an insufficient control over volume changes and losses during the procedure because the internal standard (penicillin V) was added to the final extract just before the LC–MS/MS analysis. Thus, the internal standard could not account for variable water content in the kidney samples and losses during the three sample transfer steps (a decantation step after the extraction, a transfer of almost the entire extract after the dispersive-SPE clean-up, and a filtration of the concentrated extract using a syringe filter). In our modification, the internal standard [$^{13}C_6$]sulfamethazine is added to the homogenized sample before the extraction step together with two β-lactams (penicillin V and cefadroxil), which serve for method performance controls. These two compounds are not generally used in veterinary medicine, thus can serve as quality control standards indicating potential problems in the β-lactam method performance. The use of a suitable internal standard allows working with sample aliquots rather than the entire extract, which reduces the time required for the extract concentration step. Also, the use of an aliquot in the case of the sample transfer after the dispersive SPE clean-up helps to avoid the presence of C_{18} particles in the final extract. The final extract (after the evaporation step) contains mainly water (a weak solvent), thus the presence of C_{18} particles would potentially lead to lower analyte recoveries due to their retention/partition on the sorbent.

To further simplify the procedure, we used Mini-UniPrep syringeless filters for the filtration of the final extracts instead of syringe filters. The syringeless filters consist of two parts: a chamber and a filter plunger that together form an autosampler vial that can be used for sample storage and for sample introduction using common autosamplers. The extract is transferred into the chamber. Then, the filter plunger is compressed to filter the extract and to seal the vial. Various filtration media can be used depending on the sample type. We tested two media suitable for aqueous extracts: nylon and PVDF; and found nylon to be problematic for certain analytes (lower recoveries obtained with nylon especially for amoxicillin, penicillin G, and nafcillin). Thus, PVDF (0.45 μm) was employed as a filtration medium in the syringless filters for filtration of bovine kidney extracts.

Table 2 gives β-lactam recoveries obtained at three spiking levels (10, 50, and 250 ng/g) in bovine kidney. Average recoveries were 87–103%, except for DCCD, which had an average recovery of 60%. The previous method gave average recoveries of 70–75% (58% for DCCD), thus the streamlined procedure (see Section 2.4 for the details) improved the analyte recoveries, mainly by accounting for volume changes and losses during the sample preparation. DCCD is the only free form of ceftiofur that can be monitored in a multiresidue method with other β-lactams. Upon intramuscular injection, ceftiofur metabolizes rapidly to desfuroylceftiofur, which has a free thiol group that binds *via* disulfide bonds to cysteine residues in peptides and proteins [23–25]. The lower DCCD recovery can be explained by the disulfide bond between desfuroylceftiofur and cysteine undergoing exchange with protein thiols or disulfide bonds in the kidney tissue, resulting in losses during the extraction/deproteinization step and/or nondetection in the LC–MS/MS method [4,26]. To determine total residues of ceftiofur in a single-

Table 2
β-Lactam recoveries and relative standard deviations (RSDs) obtained in bovine kidney samples fortified at 10, 50, and 250 ng/g (DCCD at 100, 500, and 2500 ng/g)

Analyte	Recovery (RSD, %)			
	10 ng/g (n = 6)	50 ng/g (n = 6)	250 ng/g (n = 6)	Overall (n = 18)
Deacetylcephapirin	100 (11)	96 (11)	98 (7)	98 (9)
Amoxicillin	88 (6)	85 (12)	87 (8)	87 (9)
DCCD	54 (12)	66 (21)	59 (5)	60 (16)
Ampicillin	91 (9)	87 (16)	89 (9)	89 (11)
Cephalexin	89 (10)	88 (14)	84 (12)	87 (11)
Cefazolin	100 (5)	103 (5)	104 (7)	102 (6)
Penicillin G	95 (7)	110 (9)	104 (10)	103 (10)
Oxacillin	98 (4)	97 (6)	102 (7)	99 (6)
Cloxacillin	97 (7)	94 (6)	102 (7)	98 (7)
Nafcillin	97 (5)	101 (7)	107 (8)	101 (8)
Dicloxacillin	96 (3)	92 (7)	102 (8)	97 (8)

residue method, desfuroylceftiofur has to be released from the disulfide bonds by their reduction, followed by stabilization of the thiol group by derivatization (e.g., acetylation) [26].

4. Conclusions

A previously reported multiresidue method for the analysis of important β-lactams in bovine kidney has been further streamlined by using an aliquot rather the entire extract and by employing syringeless filter vials for final extract filtration and storage. The recoveries have been improved by a proper control of the volume changes during the sample preparation. Based on the results of the stability study and LC mobile phase tests, MeOH has been eliminated from the entire method, including the LC–MS/MS analysis. Water, MeCN, and their mixtures can be used in the standard solution and sample preparation. The best overall LC–ESI(+)-MS/MS performance was achieved by using 0.1% FA as an additive in the mobile phase (in water and in MeCN). However due to the stability issues, FA should not be added to the final extracts prior to the LC–MS/MS analysis to match the mobile phase composition. To prevent carry-over in the LC–MS/MS analysis, the LC method was divided into two parts: one serving as an analytical method for injection of the sample and elution of the analytes and the other one, starting at a highly organic mobile phase composition (100% MeCN with 0.1% FA in our case), being dedicated for injection of a solvent (MeCN), washing of the system, and equilibration of the column to the initial conditions of the analytical method. In this way, a blank solvent is injected after each sample, but these in-between injections contribute minimally to the overall sample throughput.

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